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Conversion of Proteins to Diazeniumdiolate-Based Nitric Oxide Donors

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Abstract

Michael reaction of the methoxymethyl protected monodiazeniumdiolate of piperazine with 4-maleimidobutyric acid followed by its conversion to the *N*-hydroxy-succinimido ester produces a reagent capable of transferring the nitric oxide (NO)-donating diazeniumdiolate group to the terminal amines of the lysine residues contained in proteins. The reagent has been used to produce diazeniumdiolated bovine serum albumin (D-BSA) and diazeniumdiolated human serum albumin (D-HSA) containing 22 and 19 modified lysyl groups, respectively. Upon dissolution in pH 7.4 phosphate buffer at 37 °C these albumin derivatives gradually released all of their contained NO (approximately 40 moles per mole of protein) with initial rates of about 30-40 pmol/min/mg and half-lives on the order of 3 weeks. This methodology is now available for use in exploiting the unique specific metabolic interactions of proteins to target NO therapy to specific physiological processes in vivo.

INTRODUCTION

Every new discovery of yet another bioregulatory role for nitric oxide (NO) presents both a promising opportunity for the development of novel therapeutic agents and another barrier to be overcome in any attempt to target any such agents to a specific physiological process. Given the ever increasing number of such processes known to involve NO as well as the possible harmful effects (*1*) of this toxic chemical, it is apparent that, in those instances where an input of NO is necessary to achieve a desired clinical result, one of the key attributes of any pharmaceutical will be the ability to deliver the molecule to a highly specific site. Fortunately, virtually every life process presents a potential solution to this specificity problem in the form of the molecular recognition inherent in protein function.

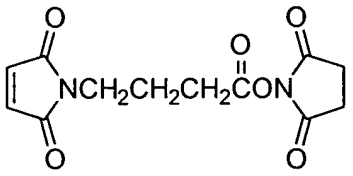
This report describes the development of a method for converting proteins into NO donors via the attachment of methoxymethylated diazeniumdiolates. The process employs several modifications of the chemistry involved in the use of the traditional cross-linking reagent *N*-(γ-maleimido-butyryloxy)succinimide (GMBS) (*2*) as well as the judicious use of protecting groups both to assemble the protein derivatizing reagent and to regulate NO release from the resulting conjugate. The method has been used to prepare serum albumin conjugates which have been studied by mass spectrometry and by analysis of their NO-releasing properties.

RESULTS AND DISCUSSION

Reagent for Diazeniumdiolate Protein Conjugation. Despite the short half-lives and acid labile nature of most diazeniumdiolates (*3*) it has proven possible to prepare versions suitable for linkage to protein-reactive reagents. As shown in Scheme 1, monoprotected piperazine can be reacted with nitric oxide to afford bifunctional compound **1**. The nitrogen in

this compound remains protected by the ethoxycarbonyl group while the diazeniumdiolate is available for alkylation by previously described methods (*4*). We chose reaction with chloromethyl methyl ether to produce the methoxymethyl (MOM) derivative **2** since this protecting group was found to hydrolyze slowly in aqueous solution at physiological pH. Removal of the ethoxycarbonyl group in refluxing ethanolic sodium hydroxide afforded amine **3**. These reactions have been described in detail elsewhere (*5*).

We envisioned a reagent which would link our diazeniumdiolate **3** to the ε-amino group of the lysines since this would be the best way to guarantee that hydrolysis to release NO would not be impeded by close proximity to the bulky protein. The widely-used heterobifunctional cross-linking reagent **6** (GMBS) (*2*) is usually utilized to link the lysyl groups of proteins (via the



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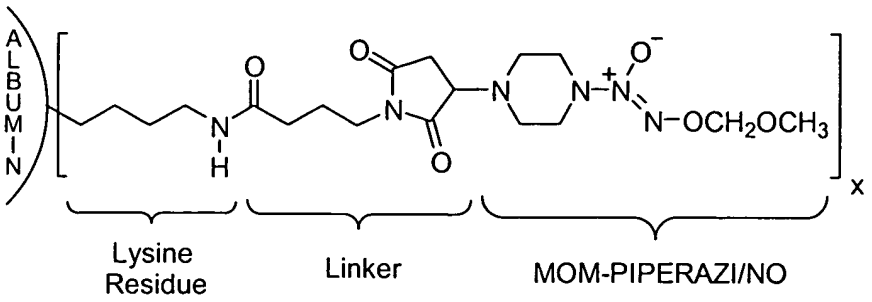
reaction of the ε-amines with the *N*-hydroxysuccinimidyl ester) with the sulfhydryl group of the same or another protein (via reaction of the thiol with the maleimide). While the reaction of the ester with the free amines of a protein is conducted without interference from the maleimide in aqueous solution at carefully controlled pH, the Michael reaction of amines with maleimides in organic solvents is well known (*6*). We used this method to prepare acid **4** which was then esterified to yield a diazeniumdiolated GMBS derivative, *N*-hydroxysuccinimide ester **5**.

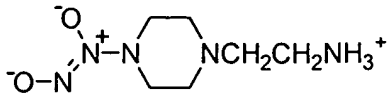
Serum Albumin Conjugates. As a demonstration of the usefulness of reagent **5** and to gain information about the properties of diazeniumdiolated proteins, we prepared diazeniumdiolated bovine serum albumin (D-BSA, **7**) and human serum albumin (D-HSA, **8**).



7: D-BSA x = ~22
8: D-HSA x = ~19

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used to compare the initial molecular weight (MW) to that of the conjugate as a means of determining the number of lysines which had reacted with the ester. Figure 1 shows the result for D-HSA. The initial MW of HSA (66.4 kDa) (*7*) was raised by 6.7 kDa which shows that an average of nineteen diazeniumdiolate-bearing sidechains (the MW of each sidechain is 356) have been added to each albumin molecule. As is customary, we employed a large excess (250 molar equivalents) of **5** in these conjugations to compensate for the hydrolysis of the reagent which competes with the desired protein reaction. It is interesting to note that while both HSA (*8*) and BSA (*9*) contain 59 lysine residues, our reagent derivatizes an average of 22 of these in the latter but only 19 in the former. We assume that the higher reactivity of the lysyl residues in BSA is in some way related to their increased degree of exposure to the proteins' surface.

Our exploration of the NO-releasing characteristics of D-BSA was begun with several assumptions based upon the small molecule chemistry already known. While the synthesis conditions required for the preparation of both reagent **5** and the protein conjugates themselves prevent the isolation of the corresponding unprotected diazeniumdiolates, the half-life of NO release in pH 7.4 phosphate buffer for the closely related piperazine **9** has been reported to be



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short (5.0 min at 22 °C) (*10*). In contrast, the rate of hydrolytic cleavage of the methoxymethyl protecting group attached to the diazeniumdiolate of compound **3** has been reported to be very slow (>17 days at pH 7.4 and 37 °C; consistent with expectations for an acetal) (*5*). This tremendous contrast in rates caused us to believe that we could observe the release of NO from these protein conjugates as a relatively simple pseudo first-order process uncomplicated by the formation of significant concentrations of intermediates. This belief was further supported by the knowledge that diazeniumdiolates such as **9** release two full equivalents of NO on decomposition with no apparent by-products other than the original amino compound (*10*). In fact, pseudo first-order kinetics has been observed even when some nitrosamine does form during diazeniumdiolate decomposition (*11*). While reports that *S*-nitros(yl)ation of proteins may serve as a transport mechanism for NO in vivo (*12*) are cause for some concern to anyone interested in analytical measurements of NO in their presence, our selection of serum albumin, a robust globular material in which all but one of the cysteine thiols are usually tied up in disulfide bonds, suggested that all of the initially released NO could be detected successfully.

With these considerations in mind, we selected an assay based upon the detection of NO by chemiluminescence which we had previously employed in a study of synthetic polymers (*13*). The release of NO was not monitored continually but rather a solution of D-BSA (**7**) in phosphate buffer at pH 7.4 was maintained at 37 °C and periodically all of the accumulated NO gas was flushed from the sample so that the rate of NO release could be measured for a period of time after the chemiluminescence trace became stabilized. Figure 2 shows the result of

monitoring the NO production of a sample of D-BSA for almost half a year. This experiment indicates that the half-life for NO release from D-BSA is on the order of 20 days. While a sample of D-HSA (**8**) has not as yet been studied over the same lengthy interval, preliminary results suggest a similar, though perhaps somewhat shorter, half-life for that protein. The area under the curve shown in Figure 2 suggests that essentially all of the 44 equivalents of NO that one would expect to be produced by hydrolytic breakdown of all 22 of the attached diazeniumdiolated piperazines are released during this experiment. Since this type of study does not lend itself conveniently to any statistical analysis to remove what can be significant variations in any single analysis for NO, we also measured the initial NO generation rate using fresh samples of D-BSA and D-HSA dissolved in the same buffer system at 37 °C. The slightly lower degree of substitution (19 vs. 22) of D-HSA vs. D-BSA was reflected in a slightly slower initial NO release rate (30 ± 5 pmol/min/mg for D-HSA (n = 2) vs. 40 ± 5 pmol/min/mg for D-BSA (n = 4)).

It should be noted that the requirement that the methoxymethyl protecting group undergo an acid-catalyzed hydrolysis as the first step toward NO release means that these protein conjugates should generate one equivalent each of formaldehyde and methanol for every two moles of NO. We have verified the increase in formaldehyde concentration with time in a solution of decomposing D-BSA but have made no attempts to quantify the production of this chemical which is both highly reactive and a ubiquitous contaminant of most laboratory equipment.

Significance. The diazeniumdiolates have emerged as an important class of NO donor compounds (*14*). The successful conjugation of these prodrugs to serum albumin will serve as a guide in the development of NO-releasing derivatives of peptide hormones, antibodies and other

proteins whose receptor-mediated interactions may be used to target NO delivery to specific points in the body. This study shows that relatively large numbers of diazeniumdiolate-containing groups can be introduced into a typical protein without detrimental consequences (although some conformational changes undoubtedly occur) and that the material can be stored for long periods of time (so far up to one year) as a solid in a freezer with no apparent decrease in activity upon reconstitution in phosphate buffer. The procedures reported herein should be applicable to diazeniumdiolates derivatized with a large variety of protecting groups which may be devised as an additional mechanism for introducing specificity into possible clinical agents based on these compounds.

Very importantly, D-HSA itself may prove to be an important clinical agent. The cardiovascular system offers many targets for NO-based therapeutic intervention (*15*) and, as a principal component of that system, serum albumin is an obvious candidate for exploitation as a drug carrier. Previous studies have shown that diazeniumdiolate-based NO donation can inhibit thrombosis (*16,17*), and D-BSA has been shown to reduce thickening of the arterial wall following coronary angioplasty in the pig (*18*). Accordingly, D-HSA may offer an excellent treatment for those cardiovascular problems that can be rectified by addition of excess NO.

EXPERIMENTAL

Nitric oxide was obtained from Matheson Gas Products (Montgomeryville, PA) and used as received. Unless noted otherwise, reagents and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI). A suitable apparatus for NO reactions has been described previously (*10*) as has the preparation of compounds **1-3** (*5*). Nuclear magnetic resonance (NMR) spectra were

recorded at 300 MHz with a Varian Unity INOVA 300WB spectrometer. Ultraviolet spectra were recorded on a Hewlett-Packard Model 8451A diode array spectrophotometer. Melting points were obtained on a hot stage and are uncorrected. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). General procedures for the handling of diazeniumdiolates have been described elsewhere (*3*) and all compounds containing this functional group are routinely stored in a freezer. Routine mass spectra were obtained with a VG Micromass 70-OPUS double focusing mass spectrometer. Protein mass spectra were obtained with a Bruker Reflex 2 MALDI-TOF mass spectrometer using a 2-mercaptobenzothiazole matrix. Nitric oxide-release rates were measured as previously described for polymeric diazeniumdiolates (*13*) except that the proteins give homogeneous solutions.

***O*²-(Methoxymethyl) 1-[4-(3'-ω-Succinimidylbutyric acid)piperazin-1-yl]diazen-1-ium-1,2-diolate (4).** A solution of 6.23 g (32.7 mmol) of **3** in 30 mL of benzene was treated with 6.00 g (32.7 mmol) of 4-maleimidobutyric acid and allowed to stir at room temperature for 18 h. The product precipitated as an off-white solid which was isolated by filtration, washed with cold benzene and then ether and dried under vacuum. Yield 9.04 g (75%); mp 139-140 °C dec; ^{1}H NMR (CD_3OD) δ 1.8-1.9 (2H, m), 2.28-2.34 (2H, m), 2.69 (1H, dd, J_1 = 5.2 Hz, J_2 = 18.2 Hz), 2.87 (1H, dd, J_1 = 8.8 Hz, J_2 = 18.2 Hz), 3.0-3.1 (2H, m), 3.45 (3H, s), 3.4-3.5 (4H, m), 3.5-3.6 (4H, m), 3.95 (1H, dd, J_1 = 5.2 Hz, J_2 = 8.8 Hz), 5.21 (2H, s); UV λ_{max} (0.1 M NaOH) 234 nm (ε = 8.28 $mM^{-1}cm^{-1}$); exact mass calcd for $C_{14}H_{24}N_5O_7$ (MH^+) 374.1676, found 374.1680.

Anal. Calcd for $C_{14}H_{23}N_5O_7$: C, 45.04; H, 6.21; N, 18.76. Found: C, 45.83; H, 6.18; N, 18.27.

***O*²-(Methoxymethyl) 1-[4-(3'-ω-Succinimidylbutyric acid)piperazin-1-yl]diazen-1-ium-1,2-diolate, *N*-Hydroxysuccinimide ester (5).** A solution of 1.617 g (14.1 mmol) of *N*-

hydroxysuccinimide in 500 mL of dry ethyl acetate was heated to just below reflux. The heat was removed and 5.25 g (14.1 mmol) of **4** was added followed by 3.20 g (15.5 mmol) of dicyclohexylcarbodiimide. The resulting mixture was stirred for 18 h, filtered and concentrated and the residual oil was dried under vacuum. Yield 6.60 g (100%); mp 56-57 °C; ^{1}H NMR ($CDCl_3$) δ 1.9-2.1 (4H, m), 2.5-2.8 (5H, m), 2.83 (4H, s), 2.8-3.1 (3H, m), 3.49 (3H, s), 3.5-3.7 (4H, m), 3.78-3.87 (1H, m), 5.22 (2H, s). UV λ_{max} (CH_3CN) 234 nm ($\varepsilon = 7.67\ mM^{-1}cm^{-1}$).

Anal. Calcd for $C_{18}H_{26}N_6O_9$: C, 45.96; H, 5.57; N, 17.86. Found: C, 46.09; H, 6.08; N, 17.90.

Diazeniumdiolated Bovine Serum Albumin. A solution of 4.00 g (0.0597 mmol) of bovine serum albumin (Calbiochem, LaJolla, CA) in 265 mL of 50 mM pH 8.5 borate buffer was treated with a solution of 6.18 g (13.1 mmol) of *N*-hydroxysuccinimide ester **5** in 23 mL of tetrahydrofuran. After stirring for 2 h, the solution was loaded into SpectraPor 4 dialysis tubing (Spectrum, Houston, TX) and dialyzed against distilled water for a total of 7 h at 0 °C during which time the 4-L reservoir of distilled water was changed 5 times. The resulting material was centrifuged to remove ca. 20 mg of residue and then freeze dried to yield 4.8 g of puffy white product. MALDI-TOF MS showed this material to have a MW of 74.2 kDa.

Sample purity was checked by capillary zone electrophoresis (CZE) employing a Beckman Model P/ACE System 5500 instrument fitted with a 50 μm ID 10% polyacrylamide-coated fused silica column with a total length of 47 cm (operative length was 40 cm). Using a mobile phase of 50 mM phosphoric acid adjusted to pH 2.5 with triethylamine, and a voltage of 12 kV (current: 16 μA), underivatized BSA eluted at 10.8 min while D-BSA eluted at 12.3 min. The D-BSA sample contained neither underivatized BSA nor any other impurity detectable by UV at 214 nm and produced a clean Gaussian-shaped peak.

Diazeniumdiolated Human Serum Albumin. A solution of 3.00 g (0.0457 mmol) of human serum albumin (Calbiochem, LaJolla, CA) in 200 mL of 50 mM pH 8.5 borate buffer was treated with a solution of 5.35 g (11.4 mmol) of *N*-hydroxysuccinimide ester **5** in 15 mL of tetrahydrofuran. After stirring for 2 h, the solution was loaded into SpectraPor 4 dialysis tubing (Spectrum, Houston, TX) and dialyzed against 50 mM pH 8.5 borate buffer for a total of 7 h at room temperature during which time the 3-L reservoir of buffer was changed 5 times. The resulting solution was centrifuged to remove ca. 20 mg of residue. The total volume of this solution was 270 mL. The solution was freeze dried to yield 4.42 g of puffy white product calculated to contain 3.24 g of diazeniumdiolated HSA and 1.18 g (26.6%) of buffer salts. MALDI-TOF MS showed this material to have a MW of 73.1 kDa.

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Legends

Scheme 1. Synthesis of Protein Diazeniumdiolation Reagent

Figure 1. MALDI-TOF mass spectra of HSA and D-HSA compared.

Figure 2. Release of NO from D-BSA as a solution in pH 7.4 phosphate buffer at 37 °C.

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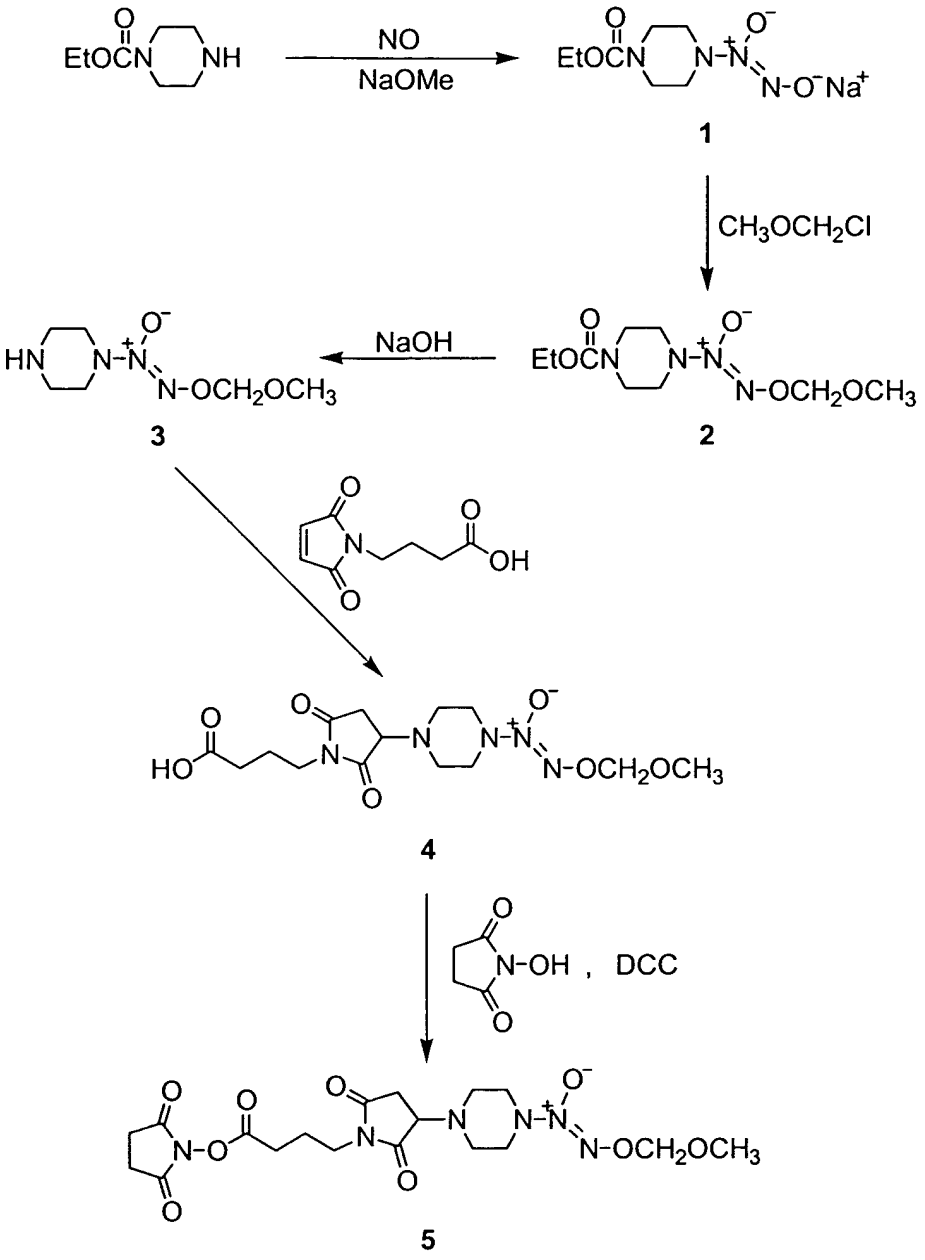

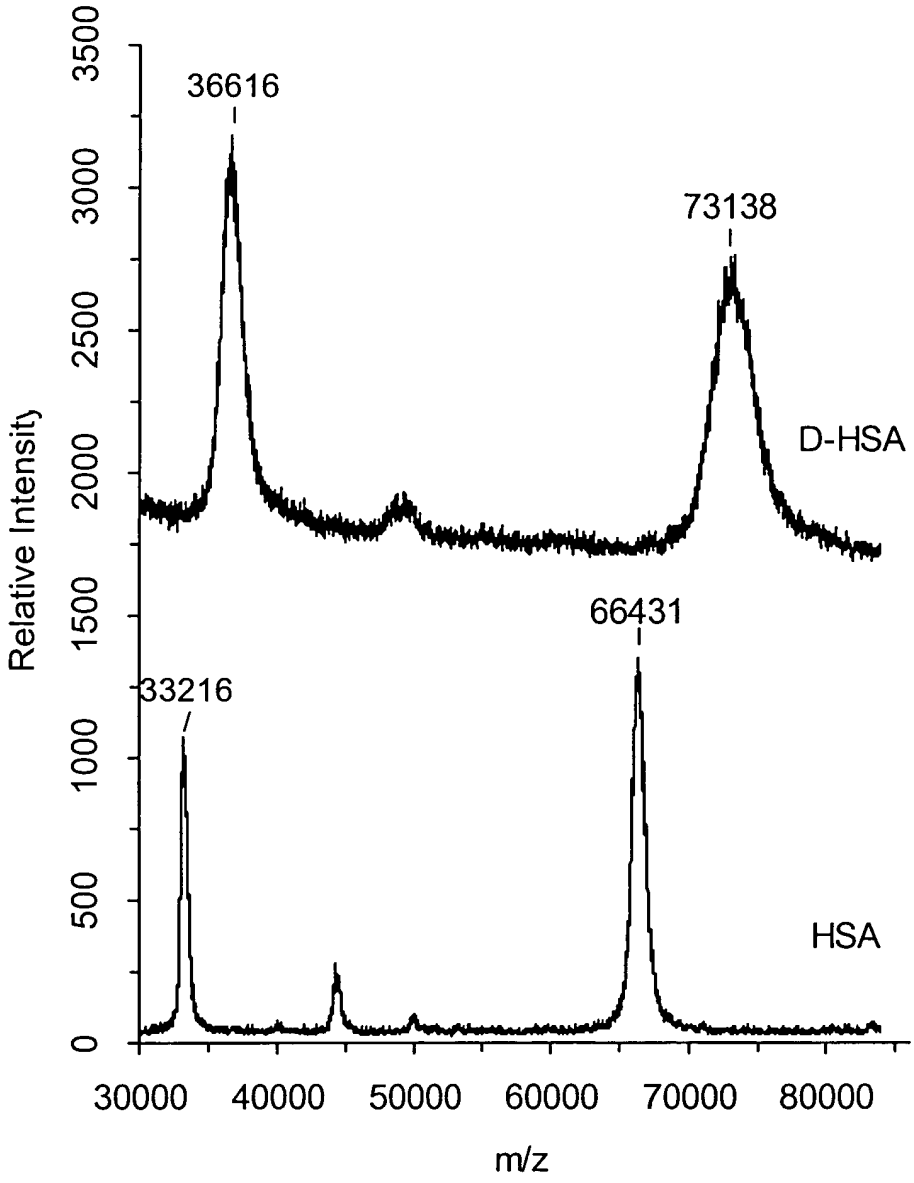

Figure 1. MALDI-TOF mass spectra of HSA and D-HSA compared.

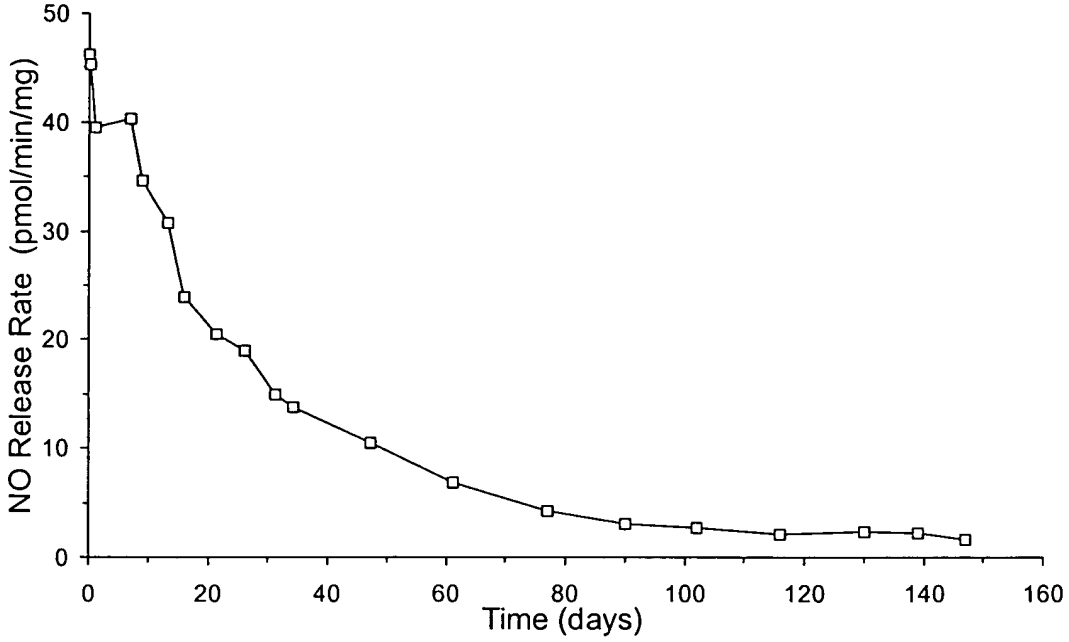

Figure 2. Release of NO from D-BSA as a solution in pH 7.4 phosphate buffer at 37 °C.